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Review

Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle

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Abstract

Corneal epithelium is traditionally thought to be a self-sufficient, self-renewing tissue implying that its stem cells are located in its basal cell layer. Recent studies indicate however that corneal epithelial stem cells reside in the basal layer of peripheral cornea in the limbal zone, and that corneal and conjunctival epithelia represent distinct cell lineages. These ideas are supported by the unique limbal/corneal expression pattern of the K3 keratin marker for corneal-type differentiation; the restriction of the slow-cycling (label-retaining) cells in the limbus; the distinct keratin expression patterns of corneal and conjunctival epithelial cells even when they are provided with identical in vivo and in vitro growth environments; and the limbal cells' superior ability as compared with central corneal epithelial cells in undergoing in vitro proliferation and in reconstituting in vivo an intact corneal epithelium. The realization that corneal epithelial stem cells reside in the limbal zone provides explanations for several paradoxical properties of corneal epithelium including its 'mature-looking' basal cells, the preponderance of tumor formation in the limbal zone, and the centripetal cellular migration. The limbal stem cell concept has led to a better understanding of the strategies of corneal epithelial repair, to a new classification of various anterior surface epithelial diseases, to the use of limbal stem cells for the reconstruction of corneal epithelium damaged or lost as a consequence of trauma or disease ('limbal stem cell transplantation'), and to the rejection of the traditional notion of 'conjunctival transdifferentiation'. The fact that corneal epithelial stem cells reside outside of the cornea proper suggests that studying corneal epithelium per se without taking into account its limbal zone will yield partial pictures. Future studies need to address the signals that constitute the limbal stem cell niche, the mechanism by which amniotic membrane facilitates limbal stem cell transplantation and ex vivo expansion, and the lineage flexibility of limbal stem cells.

Keywords: limbal epithelium; transient amplifying cells; wound repair; ocular surface disease; limbal transplantation; amniotic membrane

The corneal epithelium displays many paradoxical properties that make it unique among surface epithelia. For example, corneal epithelial basal cells are more mature looking than other stratified epithelial basal cells. The cells of the corneal epithelium undergo well-demonstrated centripetal migration, whereas other epithelia primarily exhibit vertical migration (Davanger and Evensen, 1971; Buck, 1985). The corneal epithelium almost never gives rise to tumors and, when corneal epithelial tumors do occur they are predominantly associated with the peripheral cornea in the limbal zone (a transitional area between the cornea and conjunctiva) (Waring et al., 1984). Finally, following total denudation of the corneal epithelium, it has been proposed that conjunctival epithelium can transdifferentiate into corneal epithelium (Friedenwald, 1951). Many of these puzzling corneal epithelial features can be explained through an understanding of the location and biological properties of corneal epithelial stem cells.

Although at present there are no well-proven biochemical markers of epithelial stem cells, there are several basic criteria that cells must fulfill in order to be considered stem cells. In its simplest definition, stem cells are a population of cells capable of 'unlimited' self-renewal that upon division gives rise to progeny (transient amplifying or TA cells) that have limited renewal capability (Potten and Loeffler, 1990). Additionally, stem cells divide relatively infrequently

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(slow-cycling) in mature nontraumatic tissues; however, they have a high proliferative potential (Potten, 1981). Stem cells can be induced to divide more frequently following wounding or in vitro culture conditions (Cotsarelis et al., 1989; Lavker and Sun, 2000). They are relatively small cells, which are structurally and biochemically primitive with less cellular granularity (Sun and Green, 1976; Barrandon and Green, 1985; Romano et al., 2003), and tend to be heavily pigmented in sun-exposed areas (Miller et al., 1997). Conversely, TA cells divide more frequently than stem cells and have a finite proliferative potential. Upon depletion of their proliferative capacity, TA cells undergo terminal differentiation and elaborate the specific end product(s) characteristic for the epithelium (i.e. the stratum corneum of the epidermis, the hair shaft of the follicle, the superficial cell of the corneal epithelium) (Miller et al., 1993).

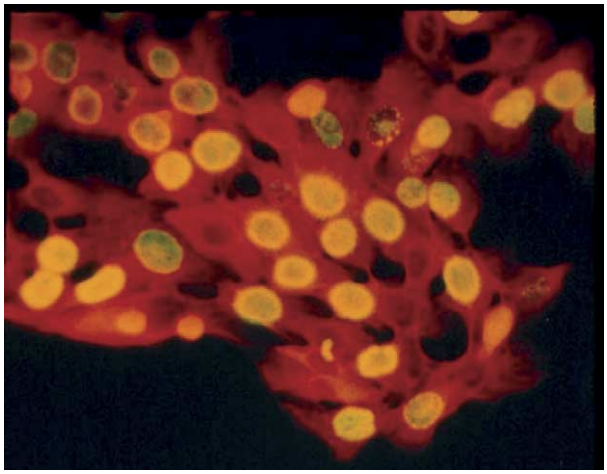


Fig. 1. Rabbit corneal epithelial cells cultured in the presence of 3T3 feeder cells, immunodouble stained with antibodies to keratin (red) and BrdU (green). Adopted from Schermer et al. (1986).

1. The limbal epithelium: a paradigm for a stem cell-enriched tissue

It is now well accepted that the limbal epithelium is the preferential site of corneal epithelial stem cells; this concept directly evolved from research on corneal epithelial growth and differentiation. Although Davanger and Evensen (1971) reported that the limbal epithelium could serve as a source of cells for the corneal epithelium, particularly after wounding, this finding could not rule out the possible migration of conjunctival cells onto the corneal epithelium proper. Thus when Thoft and Friend (1983) later proposed their 'X,Y,Z, hypothesis', they specifically stated that "while the movement of cells from the periphery of the cornea seems well established, the source of these cells were uncertain". In a later paper, Thoft (1989) recalled that "In the original X, Y, Z hypothesis, it was not necessary to ascribe an origin of the cells. Because transdifferentiation from conjunctival epithelium can occur in rabbits, it was assumed that cells from the conjunctiva could simply drift across the limbus to provide either acute or chronic replacement of peripheral corneal cells".

By performing a series of experiments analyzing the in vivo and in vitro differentiation of rabbit corneal epithelial cells (Fig. 1), Schermer et al. (1986) showed that a 64-kDa basic (K3) keratin, which can be recognized by a monoclonal antibody AE5, represents a marker for an advanced stage of corneal epithelial differentiation. This marker is expressed suprabasally in limbal epithelium but uniformly, including basal cells, in central corneal epithelium. K3 is absent from the conjunctival epithelium (Schermer et al., 1986). These results demonstrate two important points. The first is that the K3-positive limbal and corneal epithelial cells are related, and are distinct from the K3-negative conjunctival epithelium thus arguing strongly that the corneal epithelium is distinct from the conjunctival epithelium (and arguing against the concept of 'conjunctival transdifferentiation', see below). The second is that because limbal epithelial basal cells lack a marker for an advanced stage of corneal epithelial differentiation, limbal cells are biochemically more primitive than corneal basal cells. Based on these data and the centripetal migration pattern noted for corneal epithelial cells, Schermer et al. (1986) proposed that the corneal epithelial stem cells are not uniformly distributed throughout the entire corneal epithelial basal layer, but are preferentially located in the limbal epithelial basal layer (Fig. 2).

The observation that slow-cycling cells were restricted to a subset of limbal epithelial basal cells provided strong support of the limbal/corneal stem cell hypothesis (Cotsarelis et al., 1989). One of the most reliable ways to identify epithelial stem cells in vivo takes advantage of the fact that these cells are normally slow-cycling, and can be identified experimentally as 'label-retaining cells' (LRCs) (Bickenbach, 1981). This is done by long-term labeling of almost all of the cells with a DNA precursor such as tritiated thymidine or bromodeoxyuridine; this results in the labeling of all the dividing cells, including the occasionally dividing stem cells. Following a chase period, which usually lasts 4–8 weeks, the rapidly cycling TA cells lose most, if not all of their labels due to dilution, while cells that cycle slowly (the stem cells) retain the label; in this manner the stem cells can be detected as LRCs. Application of this technique to the corneal epithelium revealed that central corneal epithelium, which had been traditionally regarded as a self-sufficient, self-renewing tissue (Buschke et al., 1943; Scheving and Pauly, 1967), contained no LRCs; such cells were found exclusively in the basal layer of the peripheral corneal epithelium in the limbal zone (Fig. 3; Cotsarelis et al., 1989; Wei et al., 1995; Lehrer et al., 1998).

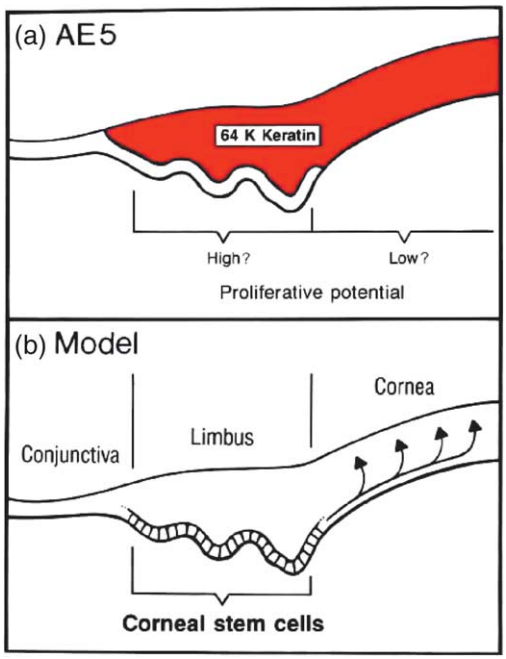


Fig. 2. The limbal stem cell concept. Panel a illustrates the expression of the K3 keratin, a marker for an advanced stage of differentiation, in the limbal and corneal epithelium. This marker is expressed suprabasally in the limbal epithelium but uniformly in the corneal epithelium. In Panel b, corneal epithelial stem cells are proposed to be located in the basal layer of the limbal epithelium. Their progeny, the TA cells, migrate centripetally towards the center of the cornea (Schermer et al., 1986). Reproduced by copyright permission of the Rockefeller Press.

Another important finding in support of the limbal stem cell concept comes from observations that limbal epithelium has greater proliferative potential than corneal epithelium. This property can best be demonstrated in vitro by growing cells from various regions of the anterior surface under identical culture conditions and subculturing them repeatedly in order to compare the proliferative potential of these separate cell populations. Thoft and co-workers showed by explant culture that corneal epithelial cells from the limbal zone grow better than peripheral and central corneal epithelium (Ebato et al., 1988). We have confirmed and extended these observations by showing that limbal cells can be subcultured more times when grown in the presence of a 3T3 feeder layer than corneal epithelial cells (Wei et al., 1993). It has been suggested that single cells in culture that form large 'holoclone' colonies are the stem cells, which are in general smaller in size (Sun and Green, 1976; Barrandon and Green, 1985; Romano et al., 2003), whereas cells forming smaller 'meroclones' and mainly abortive 'paraclones' most likely represented different stages of TA cells (Barrandon and Green, 1987). Analyzing the clonal growth properties of single human limbal and corneal basal cells, Pellegrini et al. (1999) demonstrated nicely that limbal basal cells gave rise to holoclone colonies, whereas corneal basal cells could only give rise to meroclone and paraclone colonies. In a series of in vivo experiments, we demonstrated that when limbal and corneal epithelia were continuously stimulated with phorbol myristate, limbal epithelium maintained a significantly greater proliferative response than corneal epithelium (Lavker et al., 1998), thus providing additional support to the idea that limbal cells have a greater proliferative capacity than corneal epithelial cells.

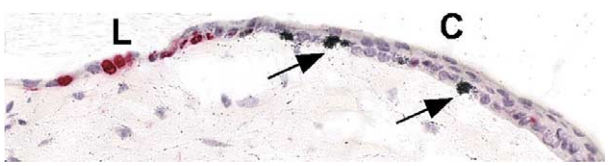


Fig. 3. Label-retaining cells are preferentially located in the basal layer of the limbal epithelium. Long-term labeling with BrdU detects LRC (red stained nuclei) and a single pulse of tritiated thymidine detects the rapidly cycling TA cells (arrows). All slow-cycling cells are preferentially located in the limbal epithelium (L). The TA cells are predominantly located in the corneal epithelium (C).

Finally, in vivo transplantation data have provided strong support for the limbal stem cell theory. Destruction of the entire corneal epithelium including its limbal stem cell zone, as occurs in chemical burn patients, leads to loss of corneal transparency (Huang and Tseng, 1991). Standard corneal replacement in such patients is ineffective because the new corneal button without an intact overlying corneal epithelium will again turn opaque. In a new surgical procedure called limbal stem cell transplantation, first described by Kenyon and Tseng (1989), small pieces of limbal epithelial tissue from either the remaining healthy eye (autograft) or from cadavers (allograft) can be used to reconstitute the entire corneal epithelium, thus allowing restoration of a healthy anterior surface (Tseng, 1989; Tseng and Sun, 1999; also see below).

Collectively these data indicate that limbal epithelium has basal cells that lack the K3 keratin and are thus biochemically undifferentiated; contains slow-cycling (label-retaining) cells; has high in vitro and in vivo proliferative potential; and can be used as an excellent source for corneal epithelial reconstruction. Conversely, the corneal epithelium has a basal cell population that is K3-positive and thus is biochemically more differentiated than the limbal basal

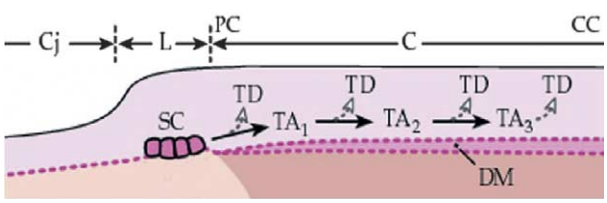


Fig. 4. A schematic diagram showing the relationship between stem cells (SC) located in the basal layer of the limbal (L) epithelium and TA cells located in the peripheral (PC) and central (CC) corneal epithelia. Solid arrows denote the well-established centripetal ('horizontal') migration of limbal-derived TA cells, which progressively lose their proliferative potential (TA_1, TA_2, TA_3) and become more mature; dashed arrows denote the ('vertical') migration of cells into the suprabasal compartment to become terminally differentiated (TD). Modified from Taylor et al. (2000).

cells; lacks slow-cycling cells; has a lower proliferative capacity than limbal epithelium; and is a poor source for corneal epithelial reconstruction. Taken together, these results strongly suggest that the corneal epithelial stem cells reside exclusively in the limbal basal layer, and that corneal epithelial basal cells represent the stem cell progeny that can be viewed as a TA cell population (Fig. 4).

2. The corneal epithelium as viewed from a limbal stem cell perspective

The limbal location of corneal epithelial stem cells can help to explain some of the peculiar properties of the corneal epithelium. First, the corneal epithelial basal cell population appears more differentiated than other epithelial basal cell populations, because corneal epithelial basal cells consist solely of TA cells with varying degrees of maturity (Lehrer et al., 1998). In other epithelia, e.g. the epidermis (Lavker and Sun, 2000), palmer and plantar epithelium (Lavker and Sun, 1982), and esophageal epithelium (Seery, 2002), the basal cell population contains both stem and TA cells, thus resulting in a more primitive appearance. Such kinetic maturity in corneal epithelial basal cells is also mirrored by biochemical maturity. For example, as discussed above, the K3/K12 keratin pair, a marker of advanced corneal epithelial differentiation, is expressed in corneal epithelial basal cells, but not in limbal epithelial basal cells (Fig. 2; Schermer et al., 1986). Likewise, a recently described calcium-linked protein (CLED) that is associated with early epithelial differentiation is expressed in corneal epithelial basal cells but not in limbal basal cells (Sun et al., 2000). S100A12, involved in Ca^{2+}-dependent signal transduction events associated with differentiated cells, is expressed throughout the corneal epithelium, but only suprabasally in the limbal epithelium (Ryan et al., 2001). Taken together, these findings indicate that corneal epithelial basal cells are biochemically equivalent to the suprabasal, differentiated cells of other, more conventional stratified squamous epithelia.

Second, an interesting and previously not well understood phenomenon is that corneal epithelial squamous cell carcinomas, which are particularly abundant in cattle, and are known as ‘cancer eye’ (Anderson, 1991), are predominantly associated with the limbus. A similar preponderance of a limbal origin of corneal epithelial tumors is known to exist in humans (Waring et al., 1984). Since stem cells are considered to be the origin of most tumors (Reya et al., 2001), and since the limbal epithelium is enriched in stem cells, it makes good biological sense that carcinomas should originate from this region.

Third, a well-established but not well understood feature of the corneal epithelium is its centripetal migration pattern, i.e. the continuous migration of cells from the limbus to central cornea (Davanger and Evensen, 1971; Buck, 1985; Lemp and Mathers, 1989). The limbal location of stem cells provides an explanation for this horizontal cellular migration. Since this migration occurs over an unusually long distance of several millimeters, corneal epithelium provides a unique system where stem cells and their progeny TA cells at different stages of maturation can be physically separated and isolated for biochemical and other studies. Thus upon division, limbal stem cells give rise to ‘young’ daughter TA cells with significant proliferative potential, which are located in the peripheral corneal epithelium (Lehrer et al., 1998). These cells migrate towards the central cornea, where they progressively lose their proliferative potential becoming more ‘mature’ TA cells (Fig. 4). TA cells of other epithelia also exhibit ‘horizontal’ migration. For example, the enterocytes of the intestinal epithelium, which are the progeny of the intestinal epithelial stem cells located in the crypts (Marshman et al., 2002), migrate along the basement membrane towards the tip of the villi. Recently, we have shown that hair follicle stem cells located in the bulge region of the follicle (Cotsarelis et al., 1990) give rise to young TA cells that can migrate downward giving rise to cells that comprise the various components of the hair shaft, as well as migrate upwards into the epidermis where they can help maintain this tissue (Taylor et al., 2000).

Fourth, the limbal stem cell concept strongly suggests that the classical concept of ‘conjunctival transdifferentiation’, a process in which conjunctival epithelial cells migrate onto a denuded corneal surface and become a bona fide corneal epithelium (Friedenwald, 1951; Shapiro et al., 1981) is incorrect. The assumption was that corneal and conjunctival epithelia were equipotent, but showed different phenotypes because of environmental modulation. Although the conjunctival epithelium can take on some aspects of the corneal phenotype when it migrates onto the corneal matrix, it does not generate a fully normal corneal epithelium (Thoft and Friend, 1977; Buck, 1986; Aitken et al., 1988; Kruse et al., 1990; Chen et al., 1994). Furthermore, recent studies have shown that rabbit forniceal conjunctival epithelial grafts when cultured on human amniotic membrane failed to transdifferentiate into corneal-type epithelial cells (Cho et al., 1999). When corneal and conjunctival epithelial cells were provided with identical in vitro and in vivo growth environments, the respective epithelial cells behaved differently. Wei et al. showed that cultured rabbit corneal and limbal epithelial cells synthesized the K3/K12 ‘corneal-specific’ keratins while bulbar, forniceal and palpebral conjunctival epithelial cells grown under the same conditions did not synthesize these keratins (Wei et al., 1993; Wu et al., 1994b). More recently, we utilized the athymic mouse/epithelial cyst model and compared the behavior of corneal/limbal epithelial cells and conjunctival epithelial cells that were placed subcutaneously into these mice (Wei et al., 1996). The epithelial cysts formed by the cultured cells reproduced/mimicked the original tissues. Thus limbal- and corneal epithelia-derived cysts were lined with glycogen-rich stratified epithelia (Fig. 5(a)). In contrast, cysts arising from cultured

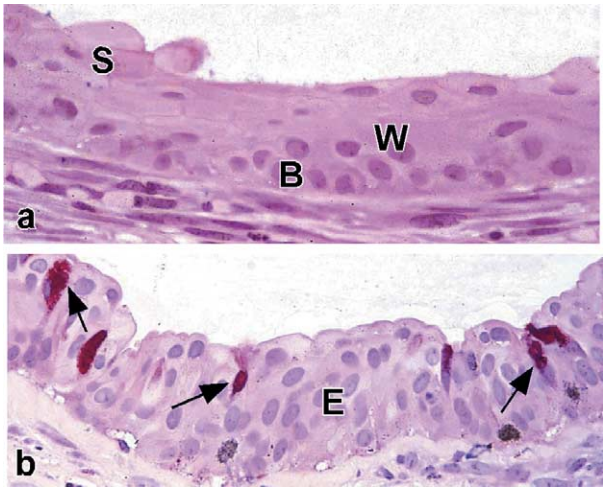


Fig. 5. Corneal/limbal lineage is distinct from the conjunctival epithelial lineage. Panel a. Stratified squamous epithelium from corneal-derived cells consists of a single layer of basal (B) cells, two to three layers of wing (W) cells and one or two layers of superficial (S) cells. Panel b. Stratified columnar epithelium from conjunctiva-derived cysts consists of several layers of columnar epithelial (E) cells interspersed with goblet cells (arrows). Since these cysts were formed under identical conditions (the stroma of the athymic mouse), the different phenotypes of these two epithelia are the result of intrinsic divergence rather than extrinsic modulation.

conjunctival epithelial cells contained PAS-positive cells with a goblet cell morphology interspersed among the stratified epithelial cells (Fig. 5(b)). Taken together, these observations provide strong evidence that the corneal/limbal epithelium and the conjunctival epithelium are intrinsically different and each is governed by its own stem cell population, and that the theory of conjunctival transdifferentiation is incorrect.

3. Basic implications of the limbal stem cell theory

3.1. Basement membrane heterogeneity and limbal stem cells

As mentioned earlier, the suprabasal expression of K3 keratin in the limbal zone, but uniform expression in corneal epithelium provided the first evidence for basal cell heterogeneity in corneal/limbal epithelium, and was largely responsible for the initial suggestion that corneal stem cells reside in the limbus (Schermer et al., 1986). Basement membrane heterogeneity seems to be responsible, at least in part, for the differential expression of the K3 gene in limbus vs. cornea. Kolega et al. (1989) showed that a monoclonal antibody AE27 stains the basement membrane of conjunctiva weakly, cornea strongly, and limbus heterogeneously. Interestingly, limbal basal cells in contact with those areas of BM that are strongly AE27-positive express K3, while those resting on BM that is AE27-negative or weak do not express K3 (Kolega et al., 1989). Additional evidence for basement membrane heterogeneity was provided by Ljubimov et al. (1995) who showed that limbal and corneal BM have different collagen type IV and laminin chain compositions. More recently, Espana et al. (2003b) showed by tissue recombination that the K3-negative phenotype of the limbal basal cells is mediated through the limbal stroma/basement membrane. Together these data suggest that the (horizontal) BM heterogeneity may play a role in regulating the expression of K3 and other differentiation-dependent genes.

3.2. Transcriptional regulation of the K3 corneal epithelial keratin gene

The regulation of K3 keratin gene was studied by isolating and characterizing a 300 bp, 5′-upstream sequence of the rabbit K3 gene which can drive reporter genes to express in keratinocytes (Wu et al., 1993). Site-directed mutagenesis and transfection assays showed that this promoter has an important motif, which can account for ~70% of the promoter activity (Wu et al., 1994a). Interestingly, this motif consists of overlapping Sp1 and AP-2 sites (Fig. 6). Occupation of the Sp1 and AP2 sites results in the activation and repression, respectively of the promoter activity. Since corneal epithelial stratification is accompanied by a 6–7 fold increase in Sp1/AP2 ratio, this can be responsible in part for the activation of the K3 promoter during corneal epithelial differentiation (Chen et al., 1997). Coupled with the previous demonstration that AP-2 activates the K14 gene in basal cells, the switch of the Sp1/AP-2 ratio during corneal epithelial differentiation may play a role in the reciprocal expression of the K3 and K14 genes in the basal and suprabasal cell layers (Chen et al., 1997).

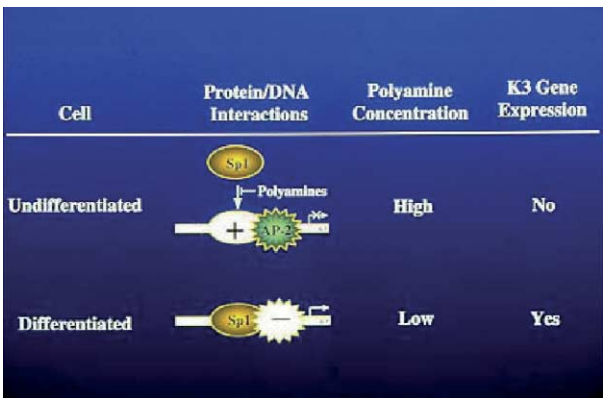


Fig. 6. Regulation of K3 gene promoter. We found a motif that can account for ~70% of a ~300 bp K3 keratinocyte-specific promoter consists of overlapping binding sites of Sp1 and AP-2 that can activate and repress, respectively, the promoter, and a greatly increased Sp1/AP-2 ratio during corneal epithelial differentiation. This finding suggests a model in which the high relative amount of AP-2 repressor, and the inhibition of Sp1 binding by polyamines that are enriched in basal cells, inhibits K3 expression. In differentiated, suprabasal cells, a greatly increased Sp1 activity and the decrease in polyamine leads to activation of K3 promoter. From Chen et al. (1997).

3.3. Corneal epithelial stem cells in development

Rodrigues et al. (1987) surveyed the changes in the K3 expression pattern in developing human corneal epithelium. It was found that at 8 weeks of gestation, the corneal epithelium consists of a single cell layer covered by periderm, and was K3-negative. At 12–13 weeks of gestation, some superficial cells of the 3–4 layered epithelium became K3-positive, providing the first sign of corneal-type differentiation. At 36 weeks, although corneal epithelium appeared morphologically mature, K3 was expressed suprabasally throughout the entire corneal/limbal epithelium (Fig. 7). These results raise the interesting possibility that all basal cells in the corneal/limbal epithelium are initially stem cells. However, basal cells in central cornea gradually acquire the expression of the K3 marker perhaps as a result of basement membrane maturation, such that in mature cornea only basal cells in the limbal zone remain to be the stem cells (Rodrigues et al., 1987). Wolosin and co-workers have shown that limbal stem cells lack connexin 43 (C43) suggesting that the absence of gap junction connection may contribute to the 'stemness' of the stem cells and may segregate the stem cells from the differentiated cells (Matic et al., 1997). They also demonstrated that the C43-negative cells may be the precursor of the stem cells (Wolosin et al., 2002). Future studies are needed to delineate the extrinsic factor(s) that may modulate corneal epithelia differentiation during embryogenesis.

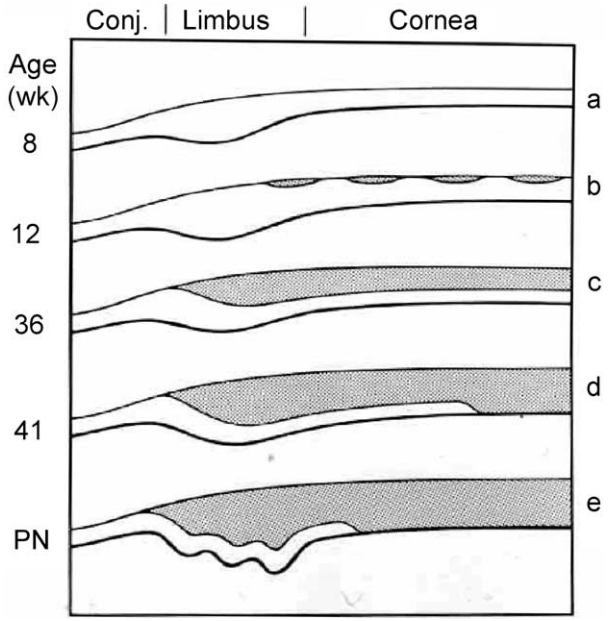


Fig. 7. Expression of K3 keratin, as assessed by AE5 staining, in human corneal epithelium during embryonic development in terms of weeks (wk) as indicated and in post-natal (PN) mature tissue. Note that all corneal basal cells are K3-negative before 36 weeks suggesting all basal cells may be stem cells at this stage of embryonic development; and the gradual transition of the basal cells to become K3-positive possibly due to basement membrane maturation such that only limbal cells remain as stem cells in post-natal, mature cornea. From Rodrigues et al. (1987).

3.4. Strategies of corneal epithelial repair

Since limbal stem cells are well separated from their progeny cells, the corneal/limbal epithelium provides an excellent model for studying the attributes of epithelial stem and TA cells (Lavker and Sun, 2000). We have investigated the in vivo growth dynamics of stem and TA cell populations in normal corneal epithelium using a double-labeling technique that permits the detection of two or more rounds of DNA synthesis in a given cell. We demonstrated that the limbal basal epithelium is heterogeneous containing both slow-cycling stem cells (detected as LRCs) as well as normally cycling TA cells (detected by pulsed-labeling) (Fig. 8(a); Lehrer et al., 1998). Following a single topical application of phorbol myristate or physical wounding of the central corneal epithelium, we observed that a large number of normally slow-cycling limbal epithelial stem cells were induced to replicate (Fig. 8(b)). The unperturbed corneal epithelial TA cell located in the peripheral region has a cell cycle time of about 72 hr, and can replicate at least twice. When induced to proliferate, the cell cycle time can be shortened to less than 24 hr and these cells can undergo additional cell divisions. In contrast, central corneal epithelial TA cells can usually divide only once prior to becoming post-mitotic even after phorbol myristate stimulation, suggesting a reduced proliferative potential. A major conclusion of this work is that a self-renewing epithelium can adopt three strategies to expand its cell population (Fig. 9), namely: (i) recruitment of the stem cells to produce more TA cells; (ii) increasing the number of times a TA cell can replicate; and/or (iii) increasing the efficiency of TA cell replication by shortening the cell cycle time (Lehrer et al., 1998).

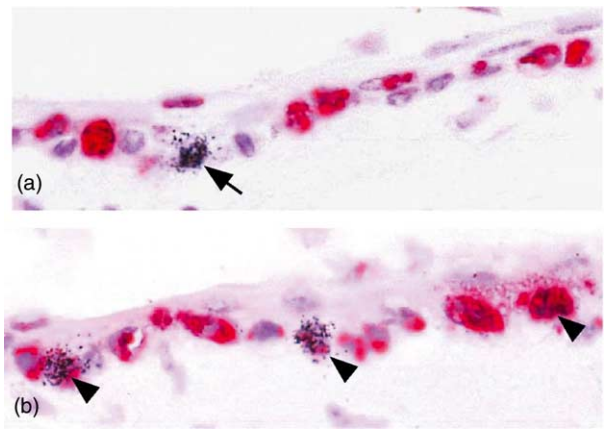


Fig. 8. Limbal stem cells can be recruited to proliferate. Under resting conditions (a) all of the slow-cycling stem cells (LRC; red stained nuclei) are located in the limbal epithelium. An occasional TA cell can also be observed among the limbal stem cells (arrow). Twenty-four hours after perturbation (b) a single pulse of tritiated thymidine was administered to mice that had populations of LRCs. Many of the LRCs are double-labeled (arrowheads) indicating that they had incorporated the tritiated thymidine and thus were undergoing a round of DNA synthesis. Modified from Lehrer et al. (1998).

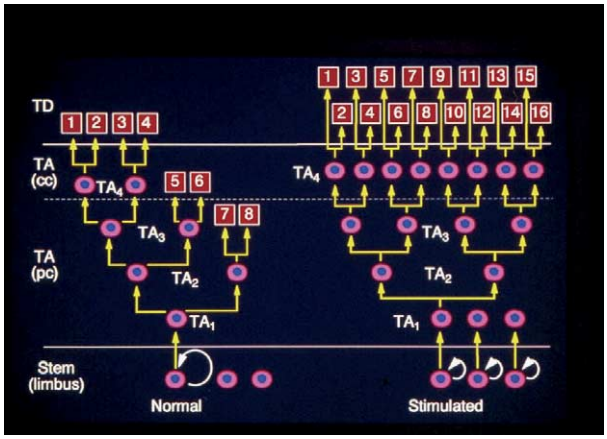


Fig. 9. Three strategies of epithelial proliferation. In the normal situation, stem cells (S) located in the limbus, cycle infrequently with a relatively long cell cycle time (large curved arrow). Upon division, stem cells give rise to regularly cycling TA cells (vertical arrows) located in the peripheral (pc) and central (cc) corneal epithelium. Young TA cells (TA1, 2, 3) are preferentially located in the peripheral cornea, whereas the more mature TA cells (TA4) reside in the central cornea and may divide only once prior to becoming terminally differentiated (TD; squares). Under normal circumstances not every TA cell will utilize its full capacity to divide, represented by those TA cells that give rise to TD cells 5–8. Upon stimulation, a self-renewing epithelium can adopt three strategies to expand its cell population. It may recruit more stem cells to divide with a more rapid cell cycle time (small curved arrows) producing more TA cells. It may induce the young TA cells (TA2, 3) to exercise their full replicative potential thereby generating more (mature) TA cells (TA4). Finally, it may increase the efficiency of TA cell replication by shortening the cell cycle time (short vertical arrows). Modified from Lehrer et al. (1998).

3.5. Cultured rabbit corneal epithelial cells as a model for wound healing

When rabbit and human corneal epithelial cells are dispersed into single cells and cultured in the presence of mitomycin C-treated, or lethally irradiated, 3T3 feeder cells, corneal keratinocytes undergo clonal growth forming a stratified epithelium (Sun and Green, 1977; Doran et al., 1980; Schermer et al., 1986). Under this condition, keratinocytes can be expanded for >10 000 folds. Normal rabbit corneal epithelium synthesizses small amounts of K5 and K14 keratins (markers for basal keratinocytes) and large amounts of K3 and K12 keratins (markers for corneal-type differentiation) (Tseng et al., 1982, 1984; Sun et al., 1984; Schermer et al., 1986). However, when corneal epithelium is wounded the cells turn off the synthesis of K3/K12 and synthesize K6 and K16 instead, keratins that are characteristic of suprabasal cells of hyperplastic stratified epithelia (Weiss et al., 1984; Jester et al., 1985; Schermer et al., 1989). Such a change in keratin expression pattern is reproduced in cultured rabbit corneal epithelial cells. These cultured cells initially express only K5 and K14 basal cell keratins, then turn on additional K6 and K16 keratins (markers for hyperproliferation), which are later replaced by K3 and K12 corneal keratins (Schermer et al., 1986, 1989). Cultured corneal epithelial cells therefore provide an excellent model for studying different stages of corneal epithelial wound repair.

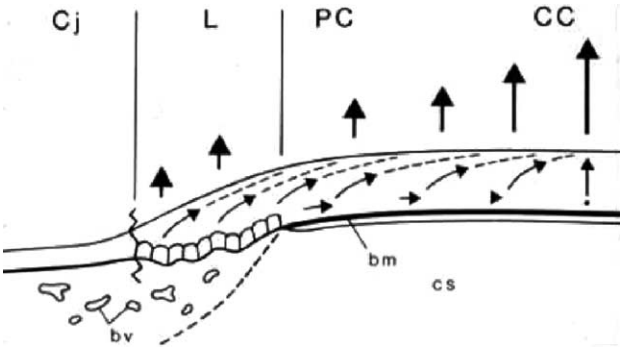


Fig. 10. The desquamation rate of superficial cells in central corneal epithelium may be higher than that of periphery thus creating a 'suction' drawing the cells to migrate centripetally. Abbreviations are Cj (conjunctiva), L (limbus), PC (peripheral cornea), CC (central cornea), bm (basement membrane), CS (corneal stroma), bv (blood vessel). See text and Lemp and Mathers (1989) and Lavker et al. (1991).

3.6. Mechanism of corneal epithelial centripetal migration

The driving force for the centripetal migration of corneal epithelial cells remains unclear. We hypothesized earlier that perhaps peripheral corneal epithelial cells, being 'younger' TA cells may proliferate faster than cells located in central cornea, thus forcing cells to migrate centripetally. This idea was compatible with some cell kinetic studies (Sharma and Coles, 1989; Lavker et al., 1991). An alternative hypothesis is that perhaps the exfoliation rate of central corneal epithelium is higher than that of peripheral corneal epithelium thus creating a 'suction', drawing peripheral cells toward the center of cornea (Fig. 10; Lemp and Mathers, 1989; Lavker et al., 1991).

4. Clinical implications of the limbal stem cell theory

The limbal stem cell theory forms the basis for identifying and reclassifying a host of corneal blinding diseases that display features of limbal stem cell deficiency (LSCD). This theory also formed the basis for the development of several surgical procedures using transplanted limbal stem cells (SC) to restore vision in patients afflicted with LSCD.

4.1. Limbal stem cell deficiency

When the limbal epithelium or the limbal stroma is damaged (Schermer et al., 1986), a pathological state termed limbal stem cell deficiency (LSCD) develops in a number of corneal diseases. Limbal deficient corneas manifest poor epithelialization (persistent defects or recurrent erosions), chronic stromal inflammation (keratitis mixed with scarring), corneal vascularization, and

Table 1
Corneal diseases manifesting LSCD

Clinical diseases	Destructive loss of limbal stem cells	Altered limbal stromal niche
I. Hereditary		
(a) Anirida		X
(b) Keratitis associated with multiple endocrine deficiency		X
(c)Epidermal dysplasia (ectrodactyly-ectodermal dysplasia-clefting syndrome)	X	
II. Acquired		
(a) Chemical or thermal burns	X	
(b) Stevens–Johnson syndrome, toxic epidermal necrolysis	X	
(c) Multiple surgeries or cryotherapies to limbus	X	
(d) Contact lens-induced keratopathy	X	
(e) Severe microbial infection extending to limbus	X	
(f) Anti-metabolite uses (5-FU or mitomycin C)	X	X
(g) Radiation	X	X
(h) Chronic limbitis (vernal, atopy, phlyctenular)		X
(i) Peripheral ulcerative keratitis (Mooren's ulcer)		X
(j) Neurotrophic keratopathy		X
(k) Chronic bullous keratopathy		X
(l) Pterygium	X	X
(m) Idiopathic	?	?

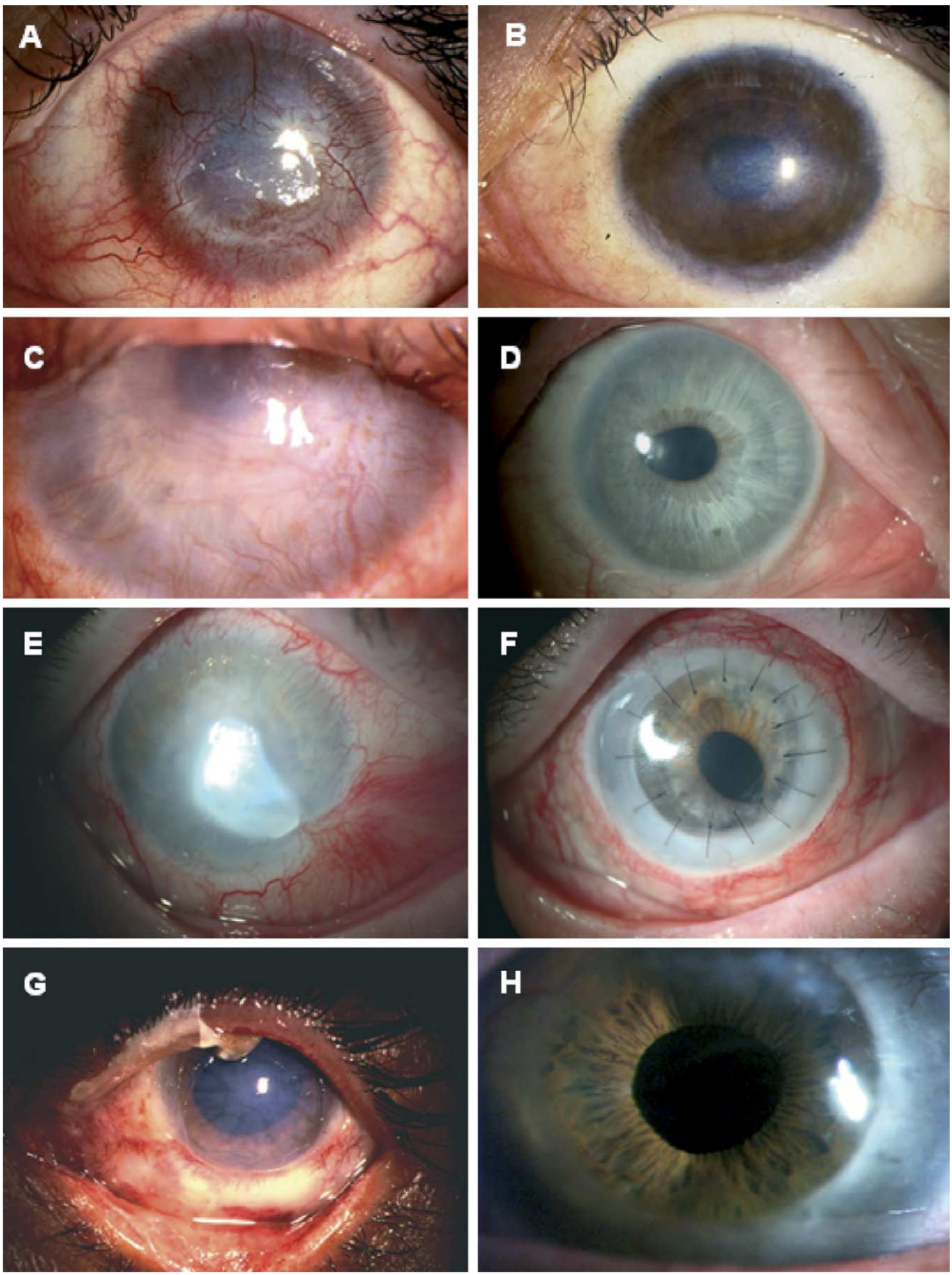
conjunctival epithelial ingrowth. Consequently, patients with LSCD experience severe irritation, photophobia, and decreased vision, making them poor candidates for conventional corneal transplantation. Because most of these clinical features can also be found in other corneal diseases, the sine qua non criterion for diagnosing LSCD is the existence of conjunctival epithelial ingrowth onto the corneal surface (i.e. conjunctivalization). Clinically, conjunctivalization may be suggested by the loss of the limbal palisades of Vogt noted under slit-lamp examination (Nishida et al., 1995), and by occurrence of late fluorescein staining (Dua et al., 1994), reflecting poor epithelial barrier function (Huang et al., 1989). However, the definitive diagnosis of conjunctivalization relies on impression cytology to detect conjunctival goblet cells on the corneal surface (Puangsricharern and Tseng, 1995). Accurate diagnosis of LSCD is crucial to choose appropriate procedures of transplanting limbal epithelial SC.

Based on the underlying etiology, corneal diseases manifesting LSCD can be subdivided into two major categories (Puangsricharern and Tseng, 1995; Table 1). In the first category, limbal epithelial stem cells are destroyed by known or recognizable offenders such as a chemical or thermal burn, Stevens–Johnson syndrome/toxic epidermal necrolysis, multiple surgeries or cryotherapies or medications (iatrogenic), contact lens, severe microbial infection, radiation, and anti-metabolites including 5-fluorouracil and mitomycin C (Puangsricharern and Tseng, 1995; Fujishima et al., 1996; Schwartz and Holland, 1998; Pires et al., 2000). A second category is characterized by a gradual loss of the stem cell population without known or identifiable precipitating factors. In this situation, the limbal stromal niche is presumably affected and progressively deteriorates by a variety of etiologies that include aniridia and coloboma, neoplasia, multiple hormonal deficiencies, peripheral ulcerative corneal diseases, neurotrophic keratopathy and idiopathic limbal deficiency (Gass, 1962; Nishida et al., 1995; Puangsricharern and Tseng, 1995; Espana et al., 2002a,b). These diseases can also be categorized according to whether inheritance is being the underlying cause, as summarized in Table 1. The underlying pathophysiology explains why transplantation of epithelial stem cells and restoration of the limbal stem cell stromal environment (e.g. by amniotic membrane transplantation) are necessary in ocular surface reconstruction. (for review see Grueterich et al., 2002a).

4.2. Transplantation of limbal epithelial stem cells

According to the type and source of tissue removed to transplant the stem cell-containing limbal epithelium, several surgical procedures have been devised. The terminology and abbreviation used herein follows the recommendation of Holland and Schwartz (1996).

Fig. 11. Clinical examples of limbal stem cell transplantation. The left panel represents the preoperative appearance of total LSCD caused by acid burn (A), Stevens–Johnson syndrome (C), alkali burn (E) and alkali burn (G). The right panel represents their corresponding post-operative appearance following CLAU (B), KLAL, amniotic membrane transplantation and extracapsular cataract extraction and lens implantation (D), KLAL, amniotic membrane transplantation, corneal transplantation, and extracapsular cataract extraction and lens implantation (F), and ex vivo expansion of limbal stem cells followed by corneal transplantation, extracapsular cataract extraction and lens implantation (H). All preoperative appearances showed characteristic features of total LSCD with reduced vision, vascularization, scarring, conjunctivalization, persistent corneal epithelial defect (A, E and G only), and band keratopathy (E only). In the first case, because the involvement was unilateral, CLAU was transplanted from the left eye to the right eye following the removal of the abnormal pannus. This resulted in improved vision with a clear and smooth cornea (photo taken 5 years later) (B). In the second case, because the involvement was bilateral, KLAL was transplanted from a cadaveric corneal limbus, resulting in improved vision with a clear and smooth cornea (photo taken 3 years later) (D). In the third case, because the involvement was bilateral, KLAL was transplanted from a cadaveric source, resulting in a smooth and healed cornea, and because the burn has caused deeper corneal scar, corneal transplantation was performed to restore vision (photo taken 1 year later) (F). The latter two cases required systemic immunosuppression. In the fourth case, because the involvement was unilateral, ex vivo expansion was performed via a small limbal biopsy, resulting in a smooth healed surface, and because of central corneal scar, corneal transplantation was performed to improve vision (photo taken 1 year later) (H).


A
B
C
D
E
F
G
H

4.3. Conjunctival limbal autograft

When total LSCD is *unilateral*, it is advised to perform conjunctival limbal autograft (CLAU), a procedure first reported by Kenyon and Tseng (1989) and experimentally confirmed by Tsai et al. (1990). Briefly, the conjunctivalized pannus is removed from the corneal surface by peritomy followed by superficial keratectomy with blunt dissection in the recipient eye. The cicatrix is removed from the subconjunctival space, invariably resulting in the recession of the conjunctival edge to 3–5 mm from the limbus. Two strips of limbal conjunctival free grafts, each spanning 6–7 mm limbal arc length, are removed by superficial lamellar keratectomy at 1 mm within the limbus from the superior and inferior limbal regions and by including 5 mm of adjacent conjunctiva. These two free grafts are transferred and secured to the recipient eye at the corresponding anatomic sites by interrupted 10-0 nylon sutures to the limbus and 8-0 vicryl sutures to the sclera. The size of the limbal zone that is removed in CLAU can be adjusted according to the visual potential of the donor eye, and the extent of LSCD in the recipient eye. The amount of the conjunctiva can be increased if the recipient eye also requires symblepharon lysis. Fig. 11(A) and (B) shows how CLAU can improve vision and restore a smooth and stable corneal surface without vascularization and without corneal transplantation in a case with unilateral acid burn. Overwhelming clinical successes have been reported by others, and collectively validate the limbal stem cell theory.

In chemical burns, severe inflammation and ischemia in the acute stage is a threat to the success of transplanted CLAU. Therefore, it is advisable to transplant amniotic membrane as a temporary patch to suppress inflammation, facilitate epithelial wound healing, and prevent scarring in acute burns (Meller et al., 2000), and Stevens–Johnson syndrome/toxic epidermal necrolysis (John et al., 2002). Although it is generally believed that the donor eyes with such limbal removal recover well without complication, scattered reports show that some donor eyes may become decompensated with pseudopterygium or partial LSCD, especially in eyes with subclinical LSCD. To preclude such a potential complication, one alternative is to transplant amniotic membrane as a graft to cover the defect after removal of CLAU in the donor eye and over the corneal surface before CLAU in the recipient eye so that the remaining and transplanted limbal epithelial SC can be expanded in the donor and recipient eye, respectively (Meallet et al., 2003).

4.4. Living-related conjunctival limbal allograft transplant (lr-CLAL)

When total LSCD is *bilateral*, corneal surface reconstruction relies on transplantation of allogeneic limbal epithelial stem cells. To do so, one option is to transplant lr-CLAL from living-related donors. The surgical procedure of lr-CLAL is identical to CLAU. Amniotic membrane can also be used similarly to eliminate the concern of removing excessive limbal epithelium from the healthy donor eye and to augment the effect of CLAU in the recipient eye (Meallet et al., 2003). Nevertheless, unless the donor and the recipient are perfectly matched, the success of lr-CLAL depends on systemic immunosuppression and allograft rejection is still the main threat (Daya and Ilari, 2001).

4.5. Keratolimbal allograft transplant (KLAL)

The other option is to perform keratolimbal allograft (KLAL) from cadaveric donors. This surgical technique was first reported by Tsai and Tseng (1994) and has since been reported by others (for a review see Espana et al., 2003a). KLAL may restore limbal stem cells in patients with bilateral LSCD or in patients with unilateral LSCD, who do not wish to expose the healthy eye to any surgical procedure including CLAU. Because of allogeneic transplantation, it is mandatory to administer systemic immunosuppression in the same manner as in lr-CLAL. Despite continuous oral administration of Cyclosporin A, Tsubota et al. (1999) and Solomon et al. (2002) reported that the long-term success of KLAL is around 40–50% in 3–5 years, while Ilari and Daya (2002) reported 21·2% of success in 5 years of follow up. The lower success rate may be attributed to severe aqueous tear deficiency dry eye (Shimazaki et al., 2000), uncorrected lid abnormalities (Solomon et al., 2002), and chronic inflammation (Schwartz et al., 2002). Because of these limiting factors, among all diseases with total LSCD, Stevens–Johnson syndrome/toxic epidermal necrolysis has the worst prognosis when treated with KLAL or lr-CLAL, because these abnormalities in the ocular surface defense collectively enhance sensitization leading to allograft rejection (Tsubota et al., 1996, 1999; Mita et al., 1999; Ilari and Daya, 2002), which remains the most important limiting factor against the success of KLAL. Signs of allograft rejection include telangiectatic and engorged limbal blood vessels, epithelial rejection lines and epithelial breakdown in severe limbal inflammation. That is why we concur with the notion that a combination of several immunosuppressive agents will have to be administered for lr-CLAL or KLAL in a manner similar to that used in other solid organ transplantations (Holland and Schwartz, 2000). Based on a new combined immunosuppressive regimen with mycophenolate, FK506 and prednisone, KLAL alone is sufficient to restore vision and a clear cornea in a patient with SJS/TENS without corneal transplantation (Fig. 11(C) and (D)). For those eyes with deeper stromal opacity or corneal edema, it is best to add corneal transplantation 3 or 4 months later when the eye is not as inflamed. In this manner vision is restored with a clear cornea (Fig. 11(E) and (F)). To further minimize corneal graft rejection, another solution is to perform deep lamellar keratoplasty rather than penetrating keratoplasty especially when there is no corneal endothelial dysfunction.

4.6. Ex vivo expansion of limbal stem cells

Another new procedure is ex vivo expansion of limbal stem cells, which was first demonstrated by Pellegrini et al. using a 3T3 fibroblast feeder layer (Pellegrini et al., 1997; Rama et al., 2001). Subsequently other investigators have used amniotic membrane with or without 3T3 fibroblast feeder layers for autologous (Chechelnitsky et al., 1999; Schwab et al., 2000; Tsai et al., 2000; Grueterich et al., 2002b) or allogeneic (Koizumi et al., 2001a,b) limbal stem cell transplantation for treating LSCD. The latter is based on the notion that amniotic membrane is an ideal substrate to restore the limbal stem cells niche for ex vivo expansion (Grueterich et al., 2003). This new surgical procedure is effective in achieving a limbal epithelial phenotype on the corneal surface (Fig. 11(G) and (H); Grueterich et al., 2002b). Clinical validity of this new surgical procedure has recently been confirmed in a long-term study in a rabbit model of unilateral total LSCD (Ti et al., 2002). The theoretical advantage of ex vivo expansion over the autologous limbal stem cell transplantation, i.e. CLAU, or living-related allogeneic limbal stem cell transplantation, i.e. lr-CLAL, is that only a small limbal biopsy is needed, thus minimizing the risk to the donor eye. The theoretical advantage over the allogeneic limbal stem cell transplantation, i.e. KLAL or lr-CLAL, is that the allograft rejection might be reduced as only epithelial cells are transplanted, and antigen-presenting Langerhan's cells are eliminated during ex vivo expansion. An FDA-approved clinical trial is in progress to validate this new surgical procedure. This new approach may one day allow us to develop new therapeutics based on gene therapies targeted at limbal stem cells in vitro.

5. Perspectives

Major advances have therefore been made in localizing and characterizing corneal epithelial stem cells in the limbus, and in the application of these cells for the treatment of ocular surface diseases. Many challenges still face epithelial stem cell biologists. For example, the generation of positive stem cell surface markers will greatly facilitate the physical isolation and molecular characterization of stem cells. Some of the currently available markers for limbal stem cells, e.g. p63 (Pellegrini et al., 2001), and enolase (Zieske et al., 1992) are expressed not only by limbal basal cells, but also by almost all basal cells of various stratified squamous epithelia making them unlikely to be stem cell-specific. Another important area is the characterization of the microenvironment that forms the stem cell niche. As described above, basement membrane heterogeneity undoubtedly contributes to the limbal and corneal epithelial phenotypes; however, many other mesenchymal signaling molecules are likely to be involved in maintaining the 'stemness' of stem cells. Some recent data suggest that amniotic membrane can support the replication of limbal stem cells and therefore may provide an experimental stem cell niche (Grueterich et al., 2003); more studies are needed to better understand the biochemical and cellular basis of this process. Finally, the area of stem cell flexibility has recently received a great deal of attention (Blau et al., 2001; Morrison, 2001; Seaberg and van der Kooy, 2003). Ferraris et al. (2000) showed that adult corneal epithelium, when combined with embryonic skin dermis, can gave rise to hair follicles indicating that, given appropriate signal(s), even the TA cells of central corneal epithelium can be converted to epidermis and its appendages. More studies are clearly needed to define the flexibility of corneal epithelial stem cells.

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